

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
APPLICATION FOR PATENT

**MELANOCORTIN RECEPTOR ANTAGONISTS AND
MODULATIONS OF MELANOCORTIN RECEPTOR ACTIVITY**

Inventors: Edward T. Wei
J. Mark Quillan
5 Wolfgang Sadée
Guennady Vlasov
J.K. Chang

Field of the Invention

10 The present invention relates to pharmaceutical compositions containing melanocortin receptor antagonists, and more particularly to methods for administering melanocortin receptor antagonists so as to modulate melanocortin receptor activity.

15 This invention was made with government support under Grant No. DA00091, awarded by the National Institutes of Health. The government has certain rights in this invention.

Background of the Invention

20 Melanocytes are embryonically derived from the neural crest. These cells migrate to the skin during fetal development, sit on the basal lamina of the epidermis and interdigitate with basal cells via dendrites. Melanin is produced in the Golgi apparatus of the cell and this pigment is packaged (melanosomes)
25 and delivered to keratinocytes and the hair follicle. In some cold-blooded vertebrates (frogs, fish, and reptiles), the cells synthesize melanosomes but do not pass it on to other cells. The melanosomes can move back and forth from the nucleus to the peripheral

processes (dendrites) and this mechanism of dispersion and aggregation gives these animals the ability to change skin color from dark to light or *vice versa*. In cold-blooded vertebrates melanocytes are called melanophores.

α -, β -, and γ - Melanocyte-Stimulating-Hormone (MSH) and adrenocorticotropin (ACTH) are melanocortin receptor (MCR) peptide agonists derived (in humans) from post-translational processing of pro-opiomelanocortin (POMC). MCR are seven-transmembrane domain G-protein coupled receptors first discovered in 1992. Five subtypes have been cloned and named MCR-1 to MCR-5. There is consensus that MCR-1 exists on normal and neoplastic melanocytes and activation of these receptors results in increased melanogenesis (via G-protein stimulation, cAMP accumulation and tyrosinase activation). The MCR-4 receptor is implicated in body weight regulation. For example, inventors Gu et al. describe using melanocortin-4 receptor as a target to treat body weight disorders by modulating the activity of that receptor, WO 97/47316, published December 18, 1997.

Melanoma is a tumor originating from unrestrained proliferation of melanocytes, which are pigment cells residing mainly in the epidermis. This tumor has an annual incidence in the United States of about 35,000 cases, with a mortality of approximately 7300 deaths (for 1997). The incidence of melanoma has been increasing significantly (with a 300 percent increase in the past 40 years). Currently, the lifetime risk of melanoma in the U.S. is approximately one percent. But in some countries such as Australia and New Zealand the lifetime risk is as high as 1/15 or 6.7%.

The reason for the increased incidence of this disease is uncertain but may stem from greater recreational sun exposure, especially early in life, and a just released study suggests that sunscreen does not protect against skin cancer, including melanoma. Individuals most susceptible to development of melanoma are those with fair complexions, red or blond hair, blue eyes, and freckles and who are poor tanners and easy sunburners. Other factors associated with increased risk include family history of melanoma (approximately one in ten melanoma patients have a family member with melanoma) and the presence or excess of atypical moles.

Malignant melanoma is usually first detected as a change in size or shape of a pigmented area of the skin and confirmed by histological examination of the biopsy specimen. The five-year survival for localized disease (clinical stages I and II) is about 85 percent. For clinical stage III (clinically palpable nodes that contain tumor cells), a five-year survival of about 50 percent is noted when only one node is involved and about 15 to 20 percent when four or more nodes are involved. Five-year survival for clinical disseminated disease (stage IV) is less than five percent. Fortunately, the majority of melanomas are diagnosed in clinical stages I and II and melanomas less than 0.76 mm thick are usually cured by surgical removal (five-year survival rates range from 96 to 99 percent). On the other hand, metastatic melanoma to organs such as brain, liver, and lung, is associated with survival of less than one year.

Current treatment of the disseminated disease is usually palliative to improve the quality of life. Surgical excision of a single metastasis to the lung or to accessible brain sites can be associated with prolonged survival. Radiation therapy can provide local

relief for recurrent tumors or metastatic sites. Patients who have advanced regional disease isolated to a limb may benefit from localized intra-arterial limb perfusion with chemotherapeutic agents. However, chemotherapy has a response rate of only 20 to 25 percent and rarely induces complete remission. The lack of response to traditional cancer treatments has led to many trials using agents such as retinoids, high-dosage chemotherapy with autologous bone marrow transplantation, antipigmentary agents, and antibodies conjugated to isotopes, drugs, and toxins. More recently, immunotherapy with interleukin-2 and α -interferon has been used. Marginally improved response rates have resulted from these experimental methods.

Wei and Thomas discovered that a number of dynorphin A peptides had the unusual property of preventing the increased vascular permeability of small blood vessels that occurs after tissue injury. This anti-inflammatory property of dynorphin A peptides and certain des-Tyr dynorphin A compounds and analogs is described in U.S. Patent 5,482,930, issued January 9, 1996.

Some MCR antagonists have been found in the *agouti* proteins (named after the South American rodent), which are 131 (mouse)/132 (human) amino acid proteins elaborated by hair follicle melanocytes and by brain tissue. The elaboration of *agouti*-signaling protein (ASP) determines the hair color of fur-coated animals. *Agouti* also antagonizes MCR-2 to MCR-5, but analyses of the binding coefficients do not indicate the clearcut characteristics of competitive antagonism which is found for MCR-1. (Yang et al., *Mol. Endocrinol.*, 11, pp. 274-280, 1997.) Siegrist et al. recently showed that ASP binds to the MCR-1 with an almost identical affinity to

that of α -MSH, and that it had antiproliferative action with a half-maximal effective concentration of 13 nM. Agouti protein was also found to induce MC down-regulation. They also showed that ASP will inhibit the α -MSH-stimulated growth of B16-F1 mouse melanoma cells in culture. Siegrist et al., *J. Recept. Signal. Transduct. Res.*, 17, pp. 75-98 (1997); Siegrist et al., *Biochem. Biophys. Res. Commun.*, 218, pp. 171-175 (1996). However, an agouti-related protein (ARP) which was recently cloned from non-epidermal tissues had little effect on MCR-1 at concentrations up to 100 nM, although it did cause a dose dependent inhibition for MCR-3 and MCR-4. Ollmann et al., *Science*, 278, pp. 135-138 (1997).

15 Summary of the Invention

It is an object of the present invention to provide antagonists to mammalian MCR receptors, particularly in the form of small or relatively small peptides.

20 The present invention provides a method for modulating the activity of a melanocortin receptor in which a melanocortin receptor antagonist is administered so as to be therapeutically useful in reducing the activity of melanocortin receptors. Thus, the present
25 invention provides that small MCR antagonists may be used for the therapy of melanoma as well as in other conditions where modulation of MCR may be of clinical significance. One example of such uses to modulate is in the case of cancerous melanocytes (melanoma) in which
30 MCR agonists stimulate growth and metastasis. Administration of an antagonist is useful in inhibiting the melanocytes. Another example is where the antagonist administered modulates the activity of MCR-4

receptors, so that modulation is useful in treating body weight disorders.

Thus, in an aspect of the present invention, a method of treating melanoma is provided in which a subject in need of treatment is administered a melanocortin receptor antagonist. The method inhibits melanocortin receptors on melanocytes when the melanocytes have administered thereto a melanocortin receptor antagonist having about seven amino acid residues and which is in an amount effective at concentrations of less than 250 nM so as to block the actions of α -melanocyte stimulating hormone. Antagonists of the invention include peptides selected from:

(a) Xaa-Arg-Xaa-Arg-Pro-Xaa-Xaa, such as dynorphin A(6-12), where Xaa⁶ is Arg or D-Arg, Ala or D-Ala, Xaa⁸ is Ile or Ala, Xaa¹¹ is Lys or D-Lys, and Xaa¹² is amidated Leu, D-Leu, or Ala. The Arg in the sixth and/or ninth position is preferably in the D-Arg stereoconfiguration, which substantially increases potency. Additional, particularly preferred modifications are where the peptides have an acylated amino terminus, or an anisoylated (methoxybenzoyl) N-terminus, and have an amidated carboxyl terminus (e.g. amidated Leu);

(b) a mystixin having the sequence T_N-A₁-A₂-A₃-A₄-A₅-A₆-T_C, where T_N is an amino terminal portion having a molecular weight less than about 600 daltons and is selected to convey resistance against enzymatic degradation. A preferred T_N, for example, is N-acetylated Tyr(Me) or methoxybenzoyl; A₁ is D- or L-arginine and D-lysine; A₂ is lysine or arginine; A₃ is leucine or isoleucine; A₄ is leucine, isoleucine, methionine, or valine; A₅ is methoxybenzoyl-ethyl-Gly, methoxybenzoylmethyl-D-Ala, Tyr(Me), Trp, Tyr, Leu, Lys,

Arg, 4' substituted Phe (4'F, 4'I, 4'Cl, 4'NO₂), D-His, D-Lys, D-Arg, D-Leu, D-Pro, or D-Trp; A₆ is isoleucine; with the proviso that not all of the A₁-A₆ are in the L-configuration; and T_c is isoleucineamide, D-leucineamide, D-valineamide; or

(c) is a compound having the sequence Arg-Tyr-Tyr-Arg-Trp/D-Trp-Lys, which may include the modifications as described in (a).

In another aspect of the present invention, a method of modulation, particularly in treating body weight disorders, comprising administering an antagonist selective for the MCR-4 receptor. Antagonists of the invention for the MCR-4 receptors in accordance with the invention include an agouti-related protein fragment (83-132), which is preferably amidated.

Although therapeutic uses are contemplated, particularly because there have been so few known antagonists of melanocortin receptors known to date, the antagonists herein described will also be useful in drug screening assays.

Aspects of the invention will be illustrated through inhibition of melanocyte cells in which a substance decreases the activity of a melanocortin receptor agonist (i.e. α -melanocyte stimulating hormone, or α -MSH) on frog melanophores or on mammalian cells transfected with melanocortin receptors. The inhibition constant (K_i), given in units of concentration, which is defined as the concentration of the antagonist which doubles the amount of agonist required to produce the same degree of effect, is computed; or the inhibitory concentration of the antagonist (IC₅₀), which reduces by 50% the activity of a fixed concentration of agonist (usually 200 to 600 pM of α -MSH), is obtained. Particularly preferred antagonists for therapeutic utility have value of K_i or IC₅₀ of less than 250 nM.

Brief Description of the Drawings

Figure 1 graphically illustrates the activity of an dynorphin A(6-12)-amide analog (called "SQW-5") in the form of a Schild Plot where the slope of the regression line is near unity; and,

Figure 2 graphically illustrates the antagonist activity of agouti-related protein (83-132)-amide where the agonist log-concentration-response curve is shifted to the right without any substantial change in slope or maximal, which is indicative of reversible competitive antagonism.

Detailed Description of the Preferred Embodiments

Hormone receptors are increasingly of interest as pharmaceutical targets, and the use of hormone-receptor antagonists for therapy is well-established in the treatment of breast and prostate cancers. Primary examples are tamoxifen and flutamine, which are antagonists of the steroidal estrogen and androgen receptors, respectively (Goodman and Gilman, Pharmacological Basis of Therapeutics, 1997).

We have discovered certain peptides that are antagonists against mammalian melanocortin receptors and which are useful in modulating the activity of these receptors. Thus, two of us have recently discovered that some dynorphin A peptides antagonize the human MCR-1, MCR-3, and MCR-4 receptors (Quillan and Sadée, *Pharm. Research*, 14, pp. 713-719, 1997). Thus, dynorphin A(1-13)-NH₂ was found to be the most potent of a series with an IC₅₀ of 110 to 145 nM (against 600 pM α -MSH). Further shortening of the carboxyl terminal end was found to cause reduction in antagonist potency. Analogs of [des-Tyr] dynorphin A were also tested. Dynorphin A(2-17) was found to have an IC₅₀ value of 190 nM (against 600 pM α -MSH). Dynorphin A(6-17) was also

tested for antagonist activity, but was found to be inactive. Antagonists useful in the subject invention further include dynorphin A(6-12), mystixins, an agouti-related protein (ARP) fragment, and some ligands for an orphan opioid receptor. These will now be more fully described.

Dynorphin A Fragments

We have surprisingly discovered that dynorphin A(6-12) and some variations thereof are particularly excellent antagonists of the MCR-1 receptor. Dynorphin fragments of the present invention, which include dynorphin A(6-12), have the general structure shown by Formula 1 (SEQ. ID NO:1):

FORMULA 1

Xaa-Arg-Xaa-Arg-Pro-Xaa-Xaa
 6 7 8 9 10 11 12

where Xaa⁶ is Arg or D-Arg, Ala or D-Ala, Xaa⁸ is Ile or Ala, Xaa¹¹ is Lys or D-Lys, and Xaa¹² is amidated Leu, D-Leu, or Ala. The Arg in the sixth and/or ninth position is preferably in the D-Arg stereoconfiguration, which substantially increases potency. Additional, particularly preferred modifications are where the peptides have an acylated amino terminus, or an anisoylated N-terminus, and have an amidated carboxyl terminus (e.g. amidated Leu). Of the various modifications or substituents possible with peptides in accordance with the present invention, the most potent modification appears to be the attachment of an anisoyl (methoxybenzoyl) group to the N-terminus. The position of the methoxy group on the benzene ring does not appear to be a critical determinant of activity as para, meta,

and ortho anisoyl- substituted analogs exhibited similar potencies. The p-nitrobenzoyl analog was as active as the anisoyl analogs. Other alternative modifications, such as p-methylpenylacetyl, p-toylacetyl, phenoxyacetyl, and p-aminobenzoyl derivatives, are possible.

In the case of dynorphin A(6-12), Xaa is arginine at position 6, isoleucine at position 8, lysine at position 11, and leucine at position 12. A particularly preferred peptide of the invention with good potency is sometimes referred to as "SQW-5," which is p-anisoyl-[D-Arg^{6,9}, D-Lys¹¹, D-Leu¹²] dynorphin A(6-12)-NH₂.

Fig. 1 graphically illustrates the activity a dynorphin A(6-12)-amide analog, SQW-5, which antagonizes the stimulatory effects of α -MSH on melanin dispersion in *Xenopus* frog melanophores. The presentation of data is in the form of a Schild Plot wherein the goal is to obtain a inhibitory constant (K_i) which estimates the antagonist potency of the test compound. At varying doses of the antagonist, the dose-ratio for the amount α -MSH to produce darkening of the melanophores is shifted. A regression analysis yields the K_i value at the intercept on the abscissa. The K_i value is defined as the concentration of antagonist, in this case 12 nM of SQW-5, which is sufficient to require a doubling of the concentration of α -MSH required to produce an equivalent effect. The slope of the regression line is near unity, which is indicative of reversible competitive antagonism.

Although less potent than the particularly preferred dynorphin A(6-12) analog, dynorphin A(1-13)-amide is another antagonist useful in practicing the subject invention.

Mystixins

Mystixins contain the sequence -Arg-Lys-Leu-
(Leu/Met)-X-Ile-(Leu/D-Leu)-NH₂, where X may be an
anisolyated glutamic derivative (A') or other aromatic
5 residues. U.S. Patent 5,480,869, issued January 2,
1996, inventors Wei and Thomas describes mystixins that
have anti-inflammatory properties and are useful in
inhibiting vascular leakage in injured tissue. These
mystixins have now been found to be antagonists against
10 mammalian MCR-1 receptors.

Thus, useful mystixins have the sequence T_N-A₁-
A₂-A₃-A₄-A₅-A₆-T_C, where T_N is an amino terminal portion
having a molecular weight less than about 600 daltons
and is selected to convey resistance against enzymatic
15 degradation. A preferred T_N, for example, is
N-acetylated Tyr(Me) or methoxybenzoyl; A₁ is D- or
L-arginine and D-lysine; A₂ is lysine or arginine; A₃ is
leucine or isoleucine; A₄ is leucine, isoleucine,
methionine, or valine; A₅ is methoxybenzoyl-ethyl-Gly,
20 methoxybenzoylmethyl-D-Ala, Tyr(Me), Trp, Tyr, Leu, Lys,
Arg, 4' substituted Phe (4'F, 4'I, 4'Cl, 4'NO₂), D-His,
D-Lys, D-Arg, D-Leu, D-Pro, or D-Trp; A₆ is isoleucine;
with the proviso that not all of the A₁-A₆ are in the
L-configuration; and T_C is isoleucineamide, D-leucine-
25 amide, D-valineamide.

Some other of the melanocortin receptor
antagonists of the present invention have also
previously been shown to have anti-inflammatory
properties. It is not known whether the previously
30 known anti-inflammatory properties are related to
antagonism on melanocortin receptors. Indeed, as will
be hereinafter discussed, in some instances peptides
useful in accordance with the present invention as
melanocortin receptor antagonists do not have
35 significant anti-inflammatory properties.

Agouti-Related Protein (ARP) Fragment

In practicing the present invention, we have found that the amidated carboxyl-terminal ARP fragment (83-132)-NH₂ is potently active with an inhibitory equilibrium disassociation constant (K_i) of 0.7 nM for the frog melanophore receptor. The sequence of this fragment (SEQ. ID NO:2) is:

SSRRCVRL HESCLGQQVP CCDPCATCYC RFFNAFCYCR KLGTAMNPCS RT

Human ARP and human ASP are two proteins found in tissues which have the property of antagonizing melanocortin receptors. Currently, these proteins are made by recombinant methods but the structural features of the proteins which determine MCR antagonism are not understood.

As will be further described, the peptide fragments of human ARP consisting of the amidated form of (83-132) were synthesized on an Applied Biosystem's 433A automated peptide synthesizer (Foster City, California) using para-methyl-benzylhydramine resin as the solid support and standard solid-phase F-moc methods. N-terminal F-moc amino acids were deprotected with 20% piperidine in dimethylformamide and coupled with o-benzotriazole-N,N,N',N'-tetramethyl-uronium hexafluorophosphate in 1-hydroxybenzotriazole. Peptide was cleaved from resin with hydrogen fluoride, washed with diethyl ether, extracted with water/acetonitrile, and cyclized at pH 8.5. Preparatory HPLC separations of the extracts yielded a peptide which was 99% pure by analytical HPLC. MALDI-TOF mass spectra of the linear fragments gave molecular weights which corresponded to theoretical values. As yet, the precise arrangement of disulfide bridges within ARP and its C-fragment, which contain 11 and 10 Cys, respectively, are undetermined.

In Fig. 2, the activity of ARP-(83-132)-NH₂ as an antagonist of α -MSH (0.1 nM) MC in frog melanophores is shown. The EC₅₀ of α -MSH alone is 0.03 nM. In the presence of ARP fragment, 30 nM, the EC₅₀ of α -MSH is increased to 19 nM. This parallel shift is indicative of competitive binding for the MC receptor site.

A Schild plot regression analysis of ARP (83-132)-NH₂ illustrates the potency of this peptide fragment. The K_i value of 0.21 nM is less than that of dynorphin A peptides and mystixin peptides, but comparable to the intact protein.

Ligands for an Orphan Opioid Receptor

An orphan opioid receptor (ORL1) was recently identified and its endogenous ligand characterized and called nociceptin/orphanin FQ. Nociceptin/orphanin FQ is a 17-amino acid residue peptide with structural homology to dynorphin A(1-17). C.T. Dooley, C. Spaeth, L. Toll, and R.A. Houghten presented an abstract at the 27th Meeting of the International Narcotics Research Conference, abstract TU13, Long Beach, California 1996; cited in detail in *Trends in Pharmacological Sciences*, 18, pp. 293-300 (August 1997), (article by G. Henderson and A.T. McKnight, "The Orphan Opioid Receptor and its Endogenous Ligand - Nociceptin/Orphanin FQ") in which they used combinatorial libraries of hexapeptides to screen for and to find ligands for the ORL1 receptor. A ligand was identified, Ac-Arg-Tyr-Tyr-Arg-Trp-Lys-NH₂, SEQ. ID NO:3, which exhibited affinity and agonist activity at the ORL1 receptor.

We have now found that this ligand, as well as several analogs prepared with modifications to the N-terminus thereof, exhibits activity as a melanocortin receptor antagonists. One of these ligands has a

potency similar to dynorphin A(1-13)-NH₂. These data are shown in Example 4.

EXPERIMENTAL

5 A standard procedure for evaluating MCR
antagonists is to use cells which bear such receptors
and are activated by α -MSH, and to examine the shift in
concentration-response curves to α -MSH in the presence
of the antagonists. Examples of such methods are given
in Quillan and Sadée (*Pharm. Res.*, 14, pp. 713-719,
10 1997) and Ollmann et al. (*Science*, 278, pp. 135-138,
1997).

Pure cultures of melanophores derived from
Xenopus laevis embryos have been used to study MCR.
Xenopus melanophore cell cultures are maintained in 5
15 parts Leibovitz's L-15 medium (Gibco), 2 parts FBS, 3
parts deionized-distilled H₂O, and phosphate-buffered
saline. Cultured melanophore cells retain their ability
to aggregate (lightened state) or disperse (darkened
state) cytoplasmic pigment vesicles in response to
20 hormonal stimulation, melatonin causes aggregation
whereas α -MSH, vasotocin, histamine, norepinephrine, and
serotonin cause dispersion. Concentration-response
curves were measured in melanophores by quantifying the
change in absorbance at 630 nm through a monolayer of
25 cells using a BT2000 Microplate Reader (FisherBiotech,
Pittsburgh, PA) after treatment with test compounds, and
curves were fit using a logistic equation. Initial
absorbance (A_1) was measured upon addition of test
compounds after 90 minutes pretreatment with carrier
30 alone (7 parts L-15 medium in 3 parts distilled-
deionized H₂O containing 0.5% (w/w) bovine serum
albumin), or carrier plus 1 nM melatonin, to initiate

tests from either the dispersed state (high absorbance) or an aggregated state (low absorbance), respectively.

A second method for studying MCR antagonists is to use mammalian cells stably transfected with the cDNA for various human MCR. These transfected cells manifest an increased cAMP response to α -MSH stimulation and agents which antagonize MCR will block this stimulating effect of α -MSH. The inhibitory effects of antagonists can be quantified by the change in the effective concentration (EC_{50}) of α -MSH in the presence of antagonist, or by computation of K_i value by a Schild regression analysis.

EXAMPLE 1

Dyn A(6-12) analogs demonstrate MCR antagonist activity by blocking α -MSH activation of *Xenopus* MC Receptors

Dynorphin A peptides have no direct stimulatory effect on MC receptors endogenous to *Xenopus* melanophores but inhibited activation of these receptors by α -MSH. To compare the relative potency of various dynorphin A(6-12) analogs, IC_{50} measurements were made against a fixed concentration (600 pM) of α -MSH. Melanophores were used because dynorphin peptides were of comparable potency at both frog and human MC receptors.

A graphical illustration for the determination of the antagonist potency, as measured by K_i , is shown for the analog SQW-5 (Fig. 1). The melanophores in the presence of 600 pM of α -MSH have an absorbance 200% greater than the initial value (A_1). Increasing concentrations of SQW-5 block the α -MSH effect and the increased light passing through the aggregated melanosomes is measured by the decrease in absorbance. The logistic fit of the data gives the IC_{50} value which

is the statistically most reliable point for measurement of potency.

None of the dynorphin A(6-12) analogs tested caused stimulation (darkening) of melanophores, but most were active as antagonists against α -MSH with IC_{50} values ranging from 17 to 1600 nM. The results are shown in Table 1. The more potent analogs (SQW-1, 4, 5, 6) with IC_{50} of 17 to 42 nM were active as anti-edema agents, but the correlation of anti-edema to MCR antagonist potencies was weak. For example, the good MCR antagonist potency of SQW-6, 8, and 13 was not matched by a corresponding enhanced potency in suppression of edema. In analogs singly substituted with alanine, reduction of the number of side-chain carbons in residue 11 significantly decreased MCR antagonist potency. Substitution of the L-Arg residues by the D-Arg isomer retained the MCR antagonist activity and the anti-edema potency. These analogs have not been previously described.

To further quantify the activity of candidate prototypes, dose-response curves were measured in the presence of several concentrations of antagonists and Schild plots were used to estimate equilibrium dissociation K_d values for SQW-1 and SQW-5. This analysis yielded K_i values of 27 and 12 nM, respectively, which are significantly lower than values of 71 and 79 nM found for dynorphin A(1-13)-NH₂ and dynorphin A(2-13), respectively. The Schild regression slopes for SQW-1 and SQW-5 are almost unity which indicate a mechanism of competitive inhibition at the MCR for the two analogs.

Dynorphin A(2-17) lowers blood pressure of anesthetized rats. It was therefore of interest to determine if a prototype dynorphin A(6-12) analog, with a lower molecular weight, would exhibit similar

hypotensive activity. The dose-effect curves of dynorphin A(2-17) and p-anisoysl-[D-Leu¹²] dynorphin A(6-12)-NH₂ on the mean arterial pressure of pentobarbital-anesthetized rats were compared. Analysis
5 of the integrated hypotensive activities, measured by the area-under-curve, for the two peptide, indicated that, on a molar basis, the two peptides had similar hypotensive activities. The potencies for anti-edema and hypotensive actions were not directly correlated as
10 dynorphin A(2-17) was at least twice more active than p-anisoysl-[D-Leu¹²] dynorphin A(6-12)-NH₂ as an anti-edema agent. The anti-edema activities were detectable at doses which did not produce significant hypotension.

The selectivity of the MC receptor antagonism
15 by dynorphin A(6-12) analogs was examined. Under the same experimental conditions, the dispersion of melanin initiated by norepinephrine, serotonin, and vasotocin were not inhibited by SQW-1. Peptides which inhibit heat-induced edema such as human CRH, urocortin,
20 sauvagine, xenopsin, and a neurotensin(8-13) analog, D-Arg-Arg-Pro-Trp-Ile-Leu, were relatively inactive in the frog MCR assay with IC₅₀ values of >10 μM.

TABLE 1

Melanocortin receptor antagonist activities of anisoyl-dynorphin A(6-12)-NH₂ analogs

	N-terminus	6	7	8	9	10	11	12-amide	MCR IC ₅₀ nM	Anti-Inflammatory ED ₅₀ μ moles/kg i
SQW-1	p-anisoyl-	Arg-	Arg-	Ile-	Arg-	Pro-	Lys-	D-Leu-	29 \pm 1	0.21
SQW-6	m-anisoyl-	Arg-	Arg-	Ile-	Arg-	Pro-	Lys-	Leu-	42 \pm 2	0.51
SQW-7	m-anisoyl-	Ala-	Arg-	Ile-	Arg-	Pro-	Lys-	Leu-	110 \pm 8	>1
SQW-8	m-anisoyl-	Arg-	Ala-	Ile-	Arg-	Pro-	Lys-	Leu-	75 \pm 3	>1
SQW-9	m-anisoyl-	Arg-	Arg-	Ala-	Arg-	Pro-	Lys-	Leu-	155 \pm 14	0.65
SQW-10	m-anisoyl-	Arg-	Arg-	Ile-	Ala-	Pro-	Lys-	Leu-	187 \pm 20	1.02
SQW-11	m-anisoyl-	Arg-	Arg-	Ile-	Arg-	Ala-	Lys-	Leu-	188 \pm 68	1.05
SQW-12	m-anisoyl-	Arg-	Arg-	Ile-	Arg-	Pro-	Ala-	Leu-	1600 \pm 1500	0.98
SQW-13	m-anisoyl-	Arg-	Arg-	Ile-	Arg-	Pro-	Lys-	Ala-	62 \pm 1	2.39
SQW-2	p-anisoyl-	Arg-	Arg-	Ile-	D-Arg-	Pro-	D-Lys-	D-Leu-	114 \pm 15	0.085
SQW-3	p-anisoyl-	Arg-	D-Arg-	Ile-	D-Arg-	Pro-	D-Lys-	D-Leu-	156 \pm 15	0.095
SQW-4	p-anisoyl-	D-Arg-	D-Arg-	Ile-	D-Arg-	Pro-	D-Lys-	D-Leu-	17 \pm 6	0.200
SQW-5	p-anisoyl-	D-Arg-	Arg-	Ile-	D-Arg-	Pro-	D-Lys-	D-Leu-	42 \pm 8	0.060

Anisoyl = methoxybenzoyl

* antagonism of α -MSH darkening of frog melanophores

** based on suppression of heat-induced edema in the anesthetized rat's paw

Schild Plots (concentration of the antagonist is varied and the dose-ratio (DR) computed)

SQW-1 $pK_i = 7.57 \pm 0.1$, $K_i = 27 \pm 6$ nM

SQW-5 $pK_i = 7.91 \pm 0.3$, $K_i = 12 \pm 8$ nM

5

EXAMPLE 2

Melanocortin Receptor Antagonist Activities of SQW-5 and Mystixin-7 in Cells Transfected with the Human MCR-1 and MCR-4 Receptors

Human embryonic kidney (HEK-293) cells were maintained in modified Eagle's medium (DMEM/H-16/F-12) with 10% fetal calf serum (FBS), 100 U/mL penicillin "G" and 100 μ g/mL streptomycin (P/S; Gemini). Transfections were performed using cDNA containing the human MCR-1 receptor (pcDNA1/NEO-hMC1), or MCR-4 (pcCMV-hMC4) receptors. HEK-293 cells are stably transformed with the human MCR-1 and MCR-4 receptors by electroporating 20 μ g of plasmid cDNA using a Gene Pulser transfection apparatus (450 V, 960 μ F; BioRad, Hercules, California). After six days, stable MC clonal cell lines were selected by addition of 400 μ g/ml G-418 to the medium and kept frozen for future use.

Cyclic-AMP and Concentration-Response Measurements

Peptide concentration-response measurements are made in HEK-293 cells by quantifying cAMP levels using a [8- 3 H]-cAMP kit from Amersham. Briefly, cells transfected with MC receptors, plated to confluency in 96-well tissue culture plates, are rinsed for 1 hr with 70% (vol/vol) L-15 medium containing 0.5% bovine serum albumin. After a 30 min exposure to test ligands, HEK-293 cells are rinsed twice with ice-cold 70% phosphate-buffered saline (0.07 g/L CaCl_2 , 0.07 g/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.14 g/L KH_2PO_4 , 1.51 g/L $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.14 g/L

KCl, 5.6 g/L NaCl, pH 7.4), and intracellular cAMP extracted with 1 mL of 60% (vol/vol) ethanol. Cellular debris was separated from extracted samples by centrifugation, 400 μ L aliquots of the supernatant lyophilized, and total cAMP content quantified by isotope counting.

Results

Addition of α -MSH to the transfected cells stimulates the accumulation of cyclic AMP from a basal level of about 2 pM per well to 4 to 6 pM per well. The effective concentration of α -MSH for 50% stimulation (EC_{50}) is computed in the presence or absence of the test substance -- in this case, 5 μ M of either SQW-5 or mystixin-7. These peptides produced a parallel shift to the right for the EC_{50} of α -MSH, indicating competitive antagonism at the transfected MCR-1 and MCR-4 (for SQW-5) receptors.

TABLE 2

Treatment: 5 μ M peptide antagonist	MCR-1 transfected $EC_{50} \pm SE$ nM	MCR-4 transfected $EC_{50} \pm SE$ nM
α -MSH	0.13 ± 0.08	0.6 ± 0.3
α -MSH + SQW-5	$14 \pm 5^*$	$18 \pm 6^*$
α -MSH + mystixin 7	$11 \pm 9^*$	NA

* statistically significant, $P < 0.01$

The antagonist activities of the mystixin analogs are shown in Table 3.

TABLE 3

	Mystixin 7 mer-Peptides	MCR Antagonism IC ₅₀ nM in <i>Xenopus</i>	Anti-Inflammat ry ED ₅₀ mg/kg i.v.*
	Alanine-Walk		
S-167	p-anisoyl-Ala-Lys-Leu-D-Thi-Ile-D-Leu-NH ₂	1200 ± 500	>2
S-166	p-anisoyl-Arg-Ala-Leu-Leu-D-Thi-Ile-D-Leu-NH ₂	1200 ± 1500	>2
S-160	p-anisoyl-Arg-Lys-Ala-Leu-D-Thi-Ile-D-Leu-NH ₂	197 ± 12	0.18 (0.07-0.49)
S-165	p-anisoyl-Arg-Lys-Leu-Ala-D-Thi-Ile-D-Leu-NH ₂	282 ± 39	0.046 (0.015-0.14)
S-164	p-anisoyl-Arg-Lys-Leu-Leu-D-Ala-Ile-D-Leu-NH ₂	26 ± 16	0.42 (0.18-0.96)
S-163	p-anisoyl-Arg-Lys-Leu-Leu-D-Thi-Ala-D-Leu-NH ₂	234 ± 149	0.067 (0.030-0.15)
S-162	p-anisoyl-Arg-Lys-Leu-Leu-D-Thi-Ile-D-Ala-NH ₂	43 ± 26	0.24 (0.11-0.50)
	Varlations on Residue 5		
mystixin-7 (S-21)	p-anisoyl-Arg-Lys-Leu-Leu-D-Thi-Ile-D-Leu-NH ₂	237 ± 79	0.02 (0.014-0.059)
S-114	p-anisoyl-Arg-Lys-Leu-Leu-D-Nal-Ile-D-Leu-NH ₂	166 ± 274	0.38 (0.16-0.91)
S-116	p-anisoyl-Arg-Lys-Leu-Leu-D-Tyr-Ile-D-Leu-NH ₂	77 ± 57	0.080 (0.03-0.20)
S-117	p-anisoyl-Arg-Lys-Leu-Leu-D-Trp-Ile-D-Leu-NH ₂	22 ± 13	0.16 (0.050-0.40)
S-118	p-anisoyl-Ala-Lys-Leu-Leu-D-Thi-Ile-D-Leu-NH ₂	96 ± 53	0.035 (0.012-0.10)

* Suppression of heat-induced edema in the anesthetized rat's paw

EXAMPLE 3

HEK-293 cells were maintained and transfected As described in Example 2. HEK-293 cells were stably transformed with the human MCR-1, MCR-3, and MCR-4 receptors by electroporating 20 μ g of plasmid cDNA ($\approx 8 \times 10^6$ cells per 800 μ L in 100% $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline in 0.4 cm cuvettes at 400V, and 960 μ F). After six days stable MC clonal cell lines were selected by addition of 400 μ g/mL G-418 (Geneticin; Gibco/BRL) to the medium.

Concentration response measurements were made also as described in Example 2, but in *Xenopus* fibroblasts, COS-7, HEK-293, and Y1 cells.

Dynorphin peptides were found to inhibit α -MSH-mediated activation of three recombinant human MC receptors stably expressed in HEK cells. The opioid congener dynorphin A(1-13)- NH_2 (30 μ M) produced a 300- to 1500-fold shift in concentration-response curves to α -MSH at each of the MCR-1, MCR-3, and MCR-4 receptor subtypes tested. The EC_{50} for α -MSH alone was 1.4 ± 0.6 , 4.2 ± 2.1 , and 3.0 ± 1.7 nM, for the MCR-1, MCR-3, and MCR-4 receptors respectively, and increased to $2,100 \pm 900$, $1,600 \pm 1,100$, and $2,000 \pm 1,200$ nM, respectively, in the presence of 30 μ M dynorphin A(1-13)- NH_2 . Shifts in concentration-response curves to α -MSH were not blocked by naloxone (10 μ M), indicating that opioid receptors are not involved. Dynorphin induced shifts in α -MSH response curves expressed as dose ratios were used to compare relative dynorphin effects at a number of MC and other receptor types. Dose ratios for the MCR-1, MCR-3, and MCR-4 receptors ranged from approximately 500 to 1000.

Since the non-opioid effects of dynorphin include those produced by [des-Tyr]dynorphin

metabolites, compounds which do not bind opioid receptors, we also tested dynorphin A(2-13) interactions with MC receptors. At each receptor subtype, dynorphin A(2-13) produced significant shifts in concentration-response curves that were 3 to 5 times smaller than dynorphin A(1-13)-NH₂. Dose ratios calculated from α -MSH dose-response curves measured in the presence and absence of 30 μ M dynorphin A(2-13) indicated shifts of 250 \pm 31, 128 \pm 44, and 147 \pm 13 for the MCR-1, MCR-3, and MCR-4 receptors, respectively. Therefore, dynorphin A(2-13) was an effective antagonist of α -MSH, but was of slightly lower potency than dynorphin A(1-13)-NH₂.

Dynorphin A(1-13)-NH₂ and dynorphin A(2-13) were also tested for their ability to inhibit the ACTH (MCR-2) receptor endogenous to murine Y1 adrenocortical cells. At 30 μ M, dynorphin A(2-13) and dynorphin A(1-13)-NH₂ shifted the concentration-response curve of ACTH-(1-24) approximately 15 and 200-fold to the right, respectively. The EC₅₀ of ACTH-(1-24) alone was 1.8 \pm 1.2 nM, and 28 \pm 13 and 364 \pm 112 nM in the presence of 30 μ M dynorphin A(2-13) and dynorphin A(1-13)-NH₂, respectively. Dynorphin's interaction with the MCR-2 receptor is significant but less potent than observed with the MCR-1, MCR-3, and MCR-4 receptors. The shift in the ACTH concentration-response curve suggests an estimated K_d for dynorphin A(1-13)-NH₂ in the submicromolar range. α -MSH by itself, up to concentrations of 10 μ M, was not effective in stimulating cAMP accumulation in Y1 cells, and dynorphin A(1-13)-NH₂ did not significantly affect basal, or inhibit forskolin (3 μ M) stimulated, cAMP production.

In Cos-7 cells, cells that contain an atypical MC receptor (27), α -MSH elevated cAMP production, but did so with relatively low potency (EC₅₀ ~ 80 nM). This EC₅₀ value was shifted ~50-fold by 30 μ M dynorphin

A(1-13)-NH₂, demonstrating that dynorphin A(1-13)-NH₂ also antagonizes the MC receptor subtype found in Cos-7 cells, but with somewhat lower potency ($K_i \sim 1 \mu\text{M}$).

EXAMPLE 4

5 As earlier described, the ligand SEQ. ID NO:3 has been identified as exhibiting affinity and agonist activity at the ORL1 receptor. This and several other analogs were prepared and tested for activity as melanocortin receptor antagonists. These are shown
10 below.

<u>Analog</u>	<u>IC₅₀ nM in Frog Melanophore Assay</u>
15 1 Ac-Arg-Tyr-Tyr-Arg-Trp-Lys-NH ₂	1050 ± 170
2 Ac-Arg-Tyr-Tyr-Arg-D-Trp-Lys-NH ₂	867 ± 85
3 p-Anisoyl-Arg-Tyr-Tyr-Arg-D-Trp-Lys-NH ₂	175 ± 27

20 All three peptides exhibited at least some activity as melanocortin receptor antagonists, with analog (3) having the best potency. Dynorphin A(1-13)-NH₂, which was also tested, had an IC₅₀ of 145 ± 14 nM.

25 The peptide antagonists of the invention may be administered in combination with a pharmaceutically acceptable carrier, such as isotonic saline, phosphate buffer solution or the like. Topical administration is feasible for those preferred peptides that are relatively small.

30 Compositions and excipients useful for the administration of small peptides through the nasal mucosa are, for example, described by U.K. patent

application no. 8719248, published February 24, 1988, applicant Sandoz Limited. Topical compositions with enhanced penetration through intact skin preferably include a potentiator, many of which are described by U.S. Patent 4,091,090, issued May 23, 1978, inventor Sipos.

The peptides form pharmaceutically acceptable salts and can be administered in salt form. Examples of suitable acids for the formation of pharmaceutically acceptable salts are hydrochloric, sulfuric, phosphoric, acetic, benzoic, citric, malonic, salicylic, malic, fumaric, succinic, tartaric, lactic, gluconic, ascorbic, maleic, fumaric, succinic, tartaric, lactic, gluconic, ascorbic, maleic, benzenesulfonic, methane- and ethanesulfonic, hydroxymethane- and hydroxyethanesulfonic. Salts may also be formed with suitable organic pharmaceutically acceptable base addition salts. These organic bases form a class whose limits are readily understood by those skilled in the art. Merely for purposes of illustration, the class may be said to include mono-, di-, and trialkylamines, such as methylamine, dimethylamine, and triethylamine; mono-, di-, or trihydroxyalkylamines such as mono-, di-, and triethanolamine; amino acids such as arginine and lysine; guanidine; N-methylglucosamine; N-methylglucamine; L-glutamine; N-methylpiperazine; morpholine; ethylenediamine; N-benzylphenethylamine; tris (hydroxymethyl)aminomethane; and the like. (See for example, "Pharmaceutical Salts," *J. Pharm. Sci.*, 66:1, pp. 1-19, 1977.)

For many applications, the peptide will preferably be formulated so as to enhance bioavailability. For example, U.S. Patent 5,514,670, issued May 7, 1996, describes emulsions with a plurality of submicron particles, a bioactive peptide, and an

aqueous continuous phase so as to enhance oral bioavailability of the peptide. Controlled release liposomal liquid pharmaceutical formulations for injection or oral administration are described in U.S. Patent 4,016,100, and other liposomal/peptide compositions are described by U.S. Patent 4,348,384. These three, just-noted patents are incorporated herein by reference.

EXAMPLE 5

10 Protein Synthesis

The protein fragment of human ARP consisting of residue (83-132) in an amidated form was synthesized on an Applied Biosystem's 433A automated peptide synthesizer (Foster City, California) using hydroxymethyl or paramethyl benzylhydramine resin as the solid support and standard solid-phase F-moc methods. N-terminal F-moc amino acids were deprotected with piperidine in dimethylformamide (20% v/v) and coupled using o-benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate in 1-hydroxybenzotriazole (1:1 molar equivalents, in a 0.5 molar excess). The protein fragment was cleaved from resin with hydrogen fluoride containing anisole (13% v/v) and dimethyl sulfide (1.7% v/v) for 45 minutes at 0°C, washed with ethyl ether, and extracted with water/acetonitrile (50% v/v). The ARP-(83-132)-NH₂ fragment was cyclized in an acetonitrile/NH₄HCO₃ solution adjusted to pH 8.5 with ammonium hydroxide for 72 hours, and extracted with Bio-Rex 70 cation exchange resin for 24 hours. The Bio-Rex resin was then rinsed with H₂O, cyclized protein eluted from the resin with 50% glacial acetic acid in H₂O (v/v), and analyzed by Pauly spray. Major fractions were purified by HPLC using a C-18 column over a

standard 100% H₂O (0.1% trifluoroacetic acid) to 100% acetonitrile (0.1% trifluoroacetic acid) gradient, rotoevaporated, and lyophilized. Samples were then analyzed using matrix assisted laser desorption ionization-time of flight (MALDI-TOF) and electrospray mass spectrometry.

Testing Synthetic ARP Fragments for Functional Antimelanotropic Activity

Synthetic human ARP fragments were assayed for antagonist activity using *Xenopus laevis* dermal melanophore cell preparations as described by Quillan et al., *Proc. Natl. Acad. Sci. USA*, 92, pp. 2894-2898 (1995), and Quillan and Sadée, *Pharm. Res.*, 14, pp. 713-719 (1997). Cultured melanophores can translocate cytoplasmic pigment vesicles in response to hormonal stimulation by a α -MSH, norepinephrine, vasotocin, pituitary adenylylcyclase activating peptide (PACAP), and serotonin. These compounds cause pigment dispersion and cell darkening. Pigment aggregation and cell lightening can be stimulated by melatonin. Melanophore responses were determined by measuring absorbance (OD) ratios taken through a monolayer of cells using a FisherBiotec BT2000 Microplate Reader (Pittsburgh, Pennsylvania). Absorbance was measured before and after a 90 minute exposure to test peptides and then expressed as percent of initial absorbance. (Measurements were taken after 60 minutes pretreatment with 10 nM melatonin to initiate readings from an aggregated state.)

Protein Synthesis and Mass Spectra Analysis

Preparatory HPLC separations of synthetic extracts yielded protein fragments which were 99% pure by analytical HPLC. MALDI-TOF mass spectrometric analysis of the linear ARP fragments gave a molecular

mass of 2984.5 daltons for (25-51) and 3282.5 daltons for (54-82), both of which correspond to expected theoretical values. The molecular mass of cyclized ARP-(83-132)-NH₂ as determined by MALDI-TOF and electrospray mass spectra was 5678.04 (SE 0.1%) and 5677.0 (SE 0.01%) daltons, respectively. These values are consistent with the calculated theoretical mass of 5676.7 daltons for protein containing a full complement of five disulfide bonds.

10 Antagonist Activity of the ARP Fragment

Synthetic ARP-(83-132)-NH₂ produced a dose-dependent suppression of α -MSH-induced dispersion of pigment in melanophore cells. The inhibitory equilibrium dissociation constant (K_i) of human ARP(83-132)-NH₂ as determined by Schild regression analysis, was 0.7 (SE 0.4) nM, which is comparable to K_i values reported for ASP and ARP purified by recombinant means. Each of the three protein fragments had no "agonist like" activity in melanophore cells; when applied to melanophores in the absence of α -MSH they did not cause pigment dispersion in the presence of 10 nM melatonin. The specificity of ARP for MC receptor-mediated response was tested by examining ARP(83-132)-NH₂ for interaction with other ligands known to induce pigment dispersion. ARP(83-132)-NH₂ (30 nM) had no effect on concentration-response curves evoked with norepinephrine, serotonin, PACAP-27, and Arg⁸-vasotocin, indicating that antagonism is selectively directed towards responses initialized by α -MSH stimulation of MC receptors.

These results demonstrate that a synthetic fragment of human ARP based on the amino acid sequence (83-132) can potently antagonize MC receptor function in *Xenopus* melanophores.

Electrospray mass spectrometry is a very accurate method of determining protein molecular mass and purity, but proteins containing disulfide bonds can produce multiply charged ions and fragment ions. This problem is accentuated with ARP(83-132)-NH₂ because of its multiple disulfide sites. Although the major peak in the electrospray mass spectrum was 5677.0 daltons, it contained a number of minor peaks not amenable to easy interpretation. None of the peaks, however, were consistent with protein containing less than five disulfide bonds; this was expected since cyclization reactions usually proceed to completion. To help better resolve the origin of the minor peaks we retested ARP(83-132)-NH₂ using MALDI-TOF mass spectrometry, a procedure less prone to fragmentation, and found only one major peak around 5678.04, which roughly corresponded to the expected theoretical mass of 5676.7 daltons. Although less precise, MALDI-TOF confirmed that minor peaks seen with the electrospray procedure were likely due to fragmentation. Thus, an ARP(83-132)-NH₂ structure containing a full complement of disulfide bonds appears to account for the biological activity observed in *Xenopus melanophores*.

It is to be understood that while the invention has been described above in conjunction with preferred specific embodiments, the description and examples are intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.